OXIDIZED FUNGAL ANTIGENS AND METHODS OF MAKING AND USING THEREOF

Technical Field

The present invention relates to oxidized fungal antigens and methods of making and using thereof. More particularly, the present invention provides a method for producing an oxidized fungal antigen in culture filtrate. The present invention also provides for the produced oxidized fungal antigens. Devices comprising such oxidized fungal antigens, methods for testing for fungal antibodies using the oxidized fungal antigens and methods for producing anti-fungal antibodies using oxidized fungal antigens are further provided. Antigen detection devices comprising anti-fungal antibodies raised against oxidized fungal antigens produced by the present methods are further provided.

Background Art

Fungal infections are common in the United States, and are often associated with significant morbidity. For example, *Candida* species are the fourth most common organisms isolated from US hospital patients (Wenzel and Pfaller, *Infect. Control Hosp. Epidemiol.*, 12:523-524 (1991)). *Histoplasma capsulatum* and *Coccidioides immitis* causes, respectively, 250,000 and 100,000 new infections annually, and 10-40% of infected persons become symptomatic (Bullock, *Histoplasma capsulatum*, In: Principles and Practice of Infectious Diseases, GL Mandell et al. (eds.), Churchill Livingstone, New York, 1995, pp. 2340-2353 and 2365-2375; and Stevens, *N. Engl. J. Med.*, 332:1077-1082 (1995)). Individuals with symptomatic *C. immitis* infection miss an average of 35 days of school or work, and incur a total of $24 million in medical expenses annually.

Many fungi cause respiratory infections that are indistinguishable on the basis of symptoms. However, it is important to accurately determine which fungus is responsible for infection due to differences in treatment regimens and incidences of complications (Bullock, *Histoplasma capsulatum*, In: Principles and Practice of Infectious Diseases, GL Mandell et al. (eds.), Churchill Livingstone, New York, 1995, pp. 2340-2353 and 2365-2375; and Galgiani, *Ann. Intern. Med.*, 130:293-300 (1999)). Because culture and direct antigen detection are problematic, antibody detection plays an important role in the

diagnosis and identification of fungal infections. The methods most commonly used to detect fungal antibodies include complement fixation (CF) and immunodiffusion (ID) (Zancope-Oliveira et al., *Clin. Diagn. Lab. Immunol.*, 1:90-93 (1994)). Both methods, however, have inherent drawbacks. ID detects antibodies to species-specific protein moieties, and is thus sensitive and highly specific; however, the assay takes 48 hours to perform, and appropriate interpretation requires highly skilled personnel. Although CF assays are highly sensitive, their performance is complex and labor-intensive, and they exhibit low specificity due to cross-reactive antibodies recognizing carbohydrate moieties common to several fungi (Zancope-Oliveira et al., *Clin. Diagn. Lab. Immunol.*, 1:90-93 (1994); and Yang et al., *Clin. Diagn. Lab. Immunol.*, 4:19-22 (1997)). Enzyme-linked immunosorbent assays (ELISA) for the detection of fungal antibodies have also been described, but these assays exhibit low specificity due to the same cross-reactive antibodies at issue in CF assays (Zartarian et al., *Am. J. Clin. Pathol.*, 207:148-153 (1997); and Kaufman et al., *J. Clin. Microbiol.*, 33:618-619 (1995)).

During the last decade, investigators using a western blot assay system found that periodate oxidation of fungal antigen preparations inactivates the cross-reactive carbohydrate moieties but does not disturb the structural integrity of the protein moieties (Zancope-Oliveira et al., *Clin. Diagn. Lab. Immunol.*, 1:90-93 (1994); Zancope-Oliveira et al., *Clin. Diagn. Lab. Immunol.*, 1:563-568 (1994); and Pizzini et al., *Clin. Diagn. Lab. Immunol.*, 6:20-23 (1999)). Thus, utilization of oxidized fungal antigens markedly increased the specificity of the western blot assay for fungal antibodies, without affecting sensitivity. However, the western blot assay described therein is not practical for most medical laboratories; many complex steps are required, including antigen preparation, gel electrophoresis, electrotransfer to nitrocellulose membranes, assay performance, and blot interpretation. The antigen was a culture filtrate, 0.45 μm filtered, 20x concentrated by ultrafiltration, dialyzed against PBS, purified by ion exchange chromatography, 10X concentrated by ultrafiltration, and finally dialyzed against PBS. Oxidizing the antigen also included several labor intensive and potentially contaminating steps: 4mg/mL antigen was exposed to periodate for 18 hours at 4°C, treated with glycerol for 15 minutes, treated with borohydrate for 2 hours, and finally dialyzed against water. Manufacturing the devices also included methods requiring sophisticated equipment and

skilled technicians: denaturing the oxidized antigen with heat and mercaptoethanol, electrophoresing with a 4% stacking gel and 7.5% resolving gel, transferring the proteins to nitrocellulose paper, blocking with buffered milk and tween, drying the paper, and cutting the paper into strips, etc.

Although attempts to utilize periodate-treated fungal antigens for specific detection of fungal antibodies in an ELISA system have been reported, these attempts were only marginally successful (Fisher et al., *Mycroses*, 40:83-90 (1997)). Binding of *H. capsulatum* antibodies to oxidized *Blastomyces dermatitidis* antigens was reduced only by about 20% when compared to binding to non-oxidized *B. dermatitidis* antigens. This limited reduction in cross-reactivity may have been due to the use of highly purified (and thus extensively manipulated) fungal antigens, periodate oxidation after the antigens had adsorbed to the plastic surface, or the use of an insufficient concentration of periodate.

Therefore, there exists a need in the art for oxidized fungal antigens with more reduced cross-reactivity. The present invention addresses this and other related needs.

Disclosure of the Invention

In one aspect, the present invention is directed to a method for producing a fungal antigen suitable for testing for an antibody to a fungus, the method comprising: a) providing a fungal antigen as a culture filtrate, the fungal antigen having not been purified by ion exchange chromatography or isoelectric focusing from the culture filtrate; and b) contacting the fungal antigen with an oxidizing agent to produce an oxidized fungal antigen suitable for testing for an antibody to the fungus. Devices comprising an oxidized fungal antigen, or a plurality of oxidized fungal antigens, produced by the above method, for testing for an anti-fungal antibody, or a plurality of anti-fungal antibodies, are also provided.

In another aspect, the present invention is directed to a method of testing for an antibody to a fungus in a sample, the method comprising: a) producing a fungal antigen suitable for testing for an antibody to a fungus, comprising providing a fungal antigen as a culture filtrate, the fungal antigen having not been purified by ion exchange chromatography or isoelectric focusing from the culture filtrate, and contacting the fungal

antigen with an oxidizing agent to produce an oxidized fungal antigen suitable for testing for an antibody to the fungus; b) contacting a sample suspected of containing an antibody to a fungus with the oxidized fungal antigen produced in step a) under suitable conditions to allow binding of the antibody, if present in the sample, to the oxidized fungal antigen; and c) assessing binding between the antibody and the oxidized fungal antigen to determine the presence and/or amount of the antibody in the sample.

In still another aspect, the present invention is directed to a method for producing an antibody to a fungal antigen, the method comprising: a) producing a fungal antigen comprising providing a fungal antigen as a culture filtrate, the fungal antigen having not been purified by ion exchange chromatography or isoelectric focusing from the culture filtrate, and contacting the fungal antigen with an oxidizing agent to produce an oxidized fungal antigen; b) delivering, to a vertebrate, the oxidized fungal antigen, in an amount sufficient to induce detectable production of an antibody to the antigen; and c) recovering the antibody from the vertebrate.

In yet another aspect, the present invention is directed to a method for producing a monoclonal antibody to a fungal antigen, the method comprising: a) producing a fungal antigen comprising providing a fungal antigen as a culture filtrate, the fungal antigen having not been purified by ion exchange chromatography or isoelectric focusing from the culture filtrate, and contacting the fungal antigen with an oxidizing agent to produce an oxidized fungal antigen; b) delivering, to a vertebrate, the oxidized fungal antigen, in an amount sufficient to induce detectable production of an antibody to the antigen; c) removing at least a portion of antibody-producing cells from the vertebrate; d) immortalizing the removed antibody-producing cells; e) propagating the immortalized antibody-producing cells; and f) harvesting monoclonal antibody produced by the immortalized antibody-producing cells. Methods of testing for a fungal antigen in a sample using the produced antibodies are also provided.

Brief Description of the Drawings

Figure 1 illustrates a *Histoplasma capsulatum* IgG ELISA test using an oxidized and a non-oxidized antigen.

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Figure 2 illustrates a *Coccidioides immitis* IgG ELISA test using an oxidized and a non-oxidized antigen.

Figure 3 illustrates a *Aspergillus fumigatus* IgG ELISA test using an oxidized and a non-oxidized antigen.

Figure 4 illustrates a *Blastomyces dermatitidis* IgG ELISA test using an oxidized and a non-oxidized antigen.

Modes of Carrying Out the Invention

For clarity of disclosure, and not by way of limitation, the detailed description of the invention is divided into the subsections that follow.

A.   **Definitions**

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as is commonly understood by one of ordinary skill in the art to which this invention belongs. All patents, applications, published applications and other publications referred to herein are incorporated by reference in their entirety. If a definition set forth in this section is contrary to or otherwise inconsistent with a definition set forth in the patents, applications, published applications and other publications that are herein incorporated by reference, the definition set forth in this section prevails over the definition that is incorporated herein by reference.

As used herein, "a" or "an" means "at least one" or "one or more."

As used herein, "fungus" refers to a division of eucaryotic organisms that grow in irregular masses, without roots, stems, or leaves, and are devoid of chlorophyll or other pigments capable of photosynthesis. Each organism (thallus) is unicellular to filamentous, and possesses branched somatic structures (hyphae) surrounded by cell walls containing glucan or chitin or both, and containing true nuclei.

As used herein, "culture" refers to a growth of living cells or microorganisms, *e.g.*, fungi, in a controlled artificial environment.

As used herein, "filtration" refers to a process of separating particulate matter from a fluid, such as air or liquid, by passing the fluid carrier through a medium that will not pass the particulates.

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As used herein, "filtrate" refers to a fluid, *e.g.*, a liquid, that passes through the medium in filtration.

As used herein, "a fungal antigen as a culture filtrate" refers to a culture filtrate of a fungus containing the fungal antigenic substances. The culture filtrate can contain secreted extracellular fungal antigenic substances derived from supernatant of the fungal culture. Alternatively, culture filtrate can contain intracellular fungal antigenic substances derived from solution or suspension of disrupted fungal cells.

As used herein, "fungal antigen having (or has) not been purified" means that a particular fungal antigen (or a group of fungal antigens) has not been isolated or purified from other antigenic substances in the culture filtrate by any isolation or purification methods. However, the fungal antigen existing in the culture filtrate can be concentrated or diluted along with the concentration or dilution of the culture filtrate.

As used herein, "fungal antigen having not been purified by ion exchange chromatography or isoelectric focusing from the culture filtrate" means that a particular fungal antigen (or a group of fungal antigens) has not been isolated or purified from other antigenic substances in the culture filtrate by ion exchange chromatography or isoelectric focusing. However, the fungal antigen existing in the culture filtrate can be concentrated or diluted along with the concentration or dilution of the culture filtrate, or can be isolated or purified from other antigenic substances in the culture filtrate by any isolation or purification methods other than ion exchange chromatography and isoelectric focusing.

As used herein, an "oxidizing agent (or oxidant)" refers to an agent that accepts electrons in an oxidation-reduction reaction.

As used herein the term "assessing (or assessed)" is intended to include quantitative and qualitative determination of the identity of a moiety, *e.g.*, an antigen, an antibody or an antigen-antibody complex, present in the sample, and also of obtaining an index, ratio, percentage, visual or other value indicative of the identity of a moiety in the sample. Assessment may be direct or indirect.

As used herein, "sample" refers to anything which may contain a fungal antigen or antibody to be tested by the present devices and/or methods. The sample may be a biological sample, such as a biological fluid or a biological tissue. Examples of biological fluids include urine, blood, plasma, serum, saliva, semen, stool, sputum,

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cerebral spinal fluid, tears, mucus, amniotic fluid or the like. Biological tissues are aggregates of cells, usually of a particular kind together with their intercellular substance that form one of the structural materials of a human, animal, plant, bacterial, fungal or viral structure, including connective, epithelium, muscle and nerve tissues. Examples of

5   biological tissues also include organs, tumors, lymph nodes, arteries and individual cell(s). The sample may also be a fungal culture, e.g., a fungal culture derived from a biological sample.

As used herein, a "liquid (fluid) sample" refers to a sample that naturally exists as a liquid or fluid, e.g., a biological fluid. A "liquid sample" also refers to a sample that

10   naturally exists in a non-liquid status, e.g., solid or gas, but is prepared as a liquid, fluid, solution or suspension containing the solid or gas sample material. For example, a liquid sample can encompass a liquid, fluid, solution or suspension containing a biological tissue.

15   **B.   Methods for producing oxidized fungal antigen**

In one aspect, the present invention is directed to a method for producing a fungal antigen suitable for testing for an antibody to a fungus, the method comprising: a) providing a fungal antigen as a culture filtrate, the fungal antigen having not been purified by ion exchange chromatography or isoelectric focusing from the culture filtrate;

20   and b) contacting the fungal antigen with an oxidizing agent to produce an oxidized fungal antigen suitable for testing for an antibody to the fungus.

The present methods can be used to produce an oxidized fungal antigen from any fungal genus or species. The oxidized antigens, while being reactive to antibodies to the fungus from which the fungal antigen is derived, may be reactive to antibodies to the

25   fungus of other related genus or species. Preferably, the methods will be controlled to produce oxidized fungal antigens that are able to distinguish among different fungal genus. More preferably, the methods will be controlled to produce oxidized fungal antigens that are able to distinguish among different fungal species within the same genus.

30   Fungi can be generally described as follows (Brock and Madigan, *Biology of Microorganisms (6th Ed.)*, Prentice Hall, Englewood Cliffs, New Jersey 07632 (1991),

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pp. 817-818). In contrast to the algae, the fungi lack chlorophyll. Fungi can be differentiated from bacteria by the fact that fungal cells are usually much larger and contain a nucleus, vacuoles, and mitochondria, typical of eucaryotic cells. Although the fungi are a large and diverse group of eucaryotic microorganisms, three groups of fungi

5   have major practical importance: the molds, yeasts, and mushrooms.

The habitats of fungi are quite diverse. Some are aquatic, living primarily in fresh water, and a few marine fungi are also known. Most fungi, however, have terrestrial habitats, in soil or on dead plant matter, and these types often play crucial roles in the mineralization of organic carbon in nature. A large number of fungi are parasites of

10   terrestrial plants. Indeed, fungi cause the majority of economically significant diseases of crop plants (*see* Table 1). A few fungi are parasitic on animals, including humans, although in general fungi are less significant as animal pathogens than are bacteria and viruses.

15   **Table 1.   Classification and major properties of fungi**

Group	Common name	Hyphae	Typical representatives	Type of sexual spore	Habitats	Common diseases
Ascomycetes	Sac fungi	Septate	Neurospora, Saccharomyces, Morchella (morels)	Ascospore	Soil, decaying plant material	Dutch elm, chestnut blight, ergot, rots
Basidiomycetes	Club fungi, mushrooms	Septate	Amanita (poisonous mushroom), Agaricus (edible mushroom)	Basidiospore	Soil, decaying plant material	Black stem, wheat rust, corn smut
Zygomycetes	Bread molds	Coenocytic	Mucor, Rhizopus (common bread mold)	Zygospore	Soil, decaying plant material	Food spoilage; rarely involved in parasitic disease
Oomycetes	Water molds	Coenocytic	Allomyces	Oospore	Aquatic	Potato blight, certain fish diseases

| Deuteromyce tes | Fungi imperfecti | Septate | Penicillium, Aspergillus | None | Soil, decaying plant material, surfaces of animal bodies | Plant wilt, fungal infections of animals such as ringworm, athlete's foot, and other dermatomycos es |

All fungi are organotrophs. Lacking chlorophyll, they of course cannot

photosynthesize, and the group also lacks lithotrophic forms. When compared to the

bacteria, the fungi in general have fairly simple nutritional requirements, and their

5   metabolic and biosynthetic processes are not particularly diverse or unusual. It is in their

morphological properties and in their sexual life cycles that the fungi exhibit considerable

diversity; hence it is on the basis of these characteristics that the fungi are classified.

In a specific embodiment, the present method is used to produce an oxidized

fungal antigen from a mitosporic Trichocomaceae, an Onygenaceae or a mitosporic

10   Onygenale. In another specific embodiment, the present method is used to produce an

oxidized fungal antigen from *Aspergillus*, *e.g.*, *Aspergillus fumigatus*, *Blastomyces*

(*Ajellomyces*), *e.g.*, *Blastomyces dermatitidis*, *Coccidioides*, *e.g.*, *Coccidioides immitis*,

Blastocystis, *e.g.*, *Blastocystis hominis*, *Histoplasma*, *e.g.*, *Histoplasma capsulatum* and

Histoplasma duboifii, *Candida*, *e.g.*, *Candida albicans* and *Cyrptococcus*, *e.g.*,

15   *Cyrptococcus neoformans*.

In still another specific embodiment, the present method is used to produce an

oxidized fungal antigen from a fungal antigen not derived from *Aspergillus*, *e.g.*,

Aspergillus fumigatus, *Blastomyces* (*Ajellomyces*), *e.g.*, *Blastomyces dermatitidis*,

Coccidioides, *e.g.*, *Coccidioides immitis*, *Blastocystis*, *e.g.*, *Blastocystis hominis*,

20   *Histoplasma*, *e.g.*, *Histoplasma capsulatum* and *Histoplasma duboifii*, *Candida*, *e.g.*,

Candida albicans and *Cyrptococcus*, *e.g.*, *Cyrptococcus neoformans*.

The fungal antigen to be oxidized should not have been purified by ion exchange

chromatography or isoelectric focusing from the culture filtrate. However, the fungal

antigen to be oxidized can be concentrated or diluted along with the concentration or

25   dilution of the culture filtrate, or can be isolated or purified from other antigenic

substances in the culture filtrate by any isolation or purification methods other than ion

exchange chromatography and isoelectric focusing. For example, the fungal antigen can be isolated or purified by any chromatographic methods other than ion exchange chromatography or any electrophoresis other than isoelectric focusing, as well as other types of isolation or purification methods such as centrifugation or organic-aqueous phase separations. In a specific embodiment, the fungal antigen to be oxidized has not been purified from the culture filtrate prior to the oxidation step.

The present methods can comprise additional steps. For example, the present methods can further comprise a step of concentrating the culture filtrate. The concentrating step can be conducted prior to, concurrently with and/or subsequent to the oxidation step. Preferably, the concentrating step is conducted prior to the oxidation step. In another example, the present methods can further comprise a step of enzymatically deglycosylating the fungal antigen. Any suitable deglycosylating enzymes can be used, e.g., Endo H, PNGase F, O-glycancase, etc. The enzymatic deglycosylation step can be conducted prior to, concurrently with and/or subsequent to the oxidation step. Preferably, enzymatic deglycosylation step is conducted prior to the oxidation step.

Any suitable oxidizing agent can be used in the present methods. Exemplary oxidizing agents include hydrogen peroxide (H_2O_2), ozone (O_3), polyatomic oxygen O_7, polyatomic oxygen O_8, $NaIO_4$, potassium peroxymonosulfate (oxone) (Wozniak et al., *Bioorg. Med. Chem. Lett.*, 8(19):2641-6 (1998)), D,L-S-methyllipoic acid methyl ester (Pan and Jordan, *Biochemistry*, 37(5):1357-64 (1998)), tertiary butyl hydroperoxide (Tarin et al., *Mol. Hum. Reprod.*, 2(12):895-901 (1996)), menadione (Santini et al., *Free Radic. Biol. Med.*, 20(7):915-24 (1996)), diamide (Bosin and Kasper, *J. Biochem. Toxicol.*, 7(3):139-45 (1992)), iodogen (Saha et al., *Int. J. Rad. Appl. Instrum.*, 16(4):431-3 (1989)), N-bromosuccinimide (Sinn et al., *Anal. Biochem.*, 170(1):186-92 (1988)), omeprazole (Im et al., *J. Biol. Chem.*, 260(8):4591-7 (1985)), and N-ethylmaleimide (Marzulli et al., *Boll. Soc. Ital. Biol. Sper.*, 61(1):121-7 (1985)).

In a specific embodiment, the oxidizing agent used in the present methods inactivates the cross-reactive carbohydrate moiety of the fungal antigen but does not disturb the structural integrity or antigenicity of the non-carbohydrate moieties. For example, when the fungal antigen to be oxidized is a glycosylated protein or peptide, the oxidizing agent inactivates the cross-reactive carbohydrate moiety of the fungal antigen

but does not disturb the structural integrity or antigenicity of the proteineous or peptidyl moiety.

In a preferred embodiment, the oxidizing agent used in the present methods is periodate (or periodic acid), which has a molecular formula H_5IO_6. Suitable salts, esters or other derivatives of periodate that retain periodate's oxidizing activity, *e.g.*, sodium *meta*-periodate (Sigma). can also be used. Periodate or its salts, esters or other derivatives thereof can be produced by methods known in the art. For example, periodate can be prepared by electrolytic oxidation of iodic acid or from barium periodate and nitric acid (Willard, *Inorg. Syn.* 1, 172 (1939); Chemistry of periodic acid and periodates; H. Siebert, *Fortschr. Chem. Forsch.* 8, 470 (1967); Periodic acid and periodates in organic and bioorganic chemistry; A. J. Fatiadi, *Synthesis* 1974, 229; and G. Dryhurst, *Periodate Oxidation of Diol and Other Functional Groups* (Pergamon Press, New York, 1970)). Alternatively, Periodate or its salts, esters or other derivatives thereof can be obtained commercially, *e.g.*, from Sigma.

The oxidation step can be conducted under any suitable conditions depending on a number of factors such as the fungal antigen to be oxidized, the oxidizing agent to be used, the properties of the culture filtrate, the degree of concentration and/or dilution of the culture filtrate and the presence or absence of any enzymatic deglycosylation. Generally, the concentration of the oxidizing agent can be in a wide range, *e.g.*, from 0.01 M to about 0.1 M if periodate is used. The oxidation step can be conducted at any suitable temperature, *e.g.*, from about 4°C to an ambient temperature, *e.g.*, from about 25°C to about 30°C. The incubation time can also have a wide range, *e.g.*, from about 1 hour to about 20 hours. When periodate is used, oxidation step is preferably conducted at about 25°C for about 1-3 hours.

In another specific embodiment, the present method can further comprise a step of attaching the oxidized fungal antigen to a surface of a device suitable for testing for an antibody to a fungus. Any suitable device can be used such as a microtiter plate, a glass slide, a nitrocellulose membrane, a latex bead, a cell, a test tube, a plastic bead, a colloidal gold particle, a colored particle, a magnetic bead and a quantum dot. Preferably, a microtiter plate is used.

Fungal antigens, suitable for testing for an antibody to a fungus, which are produced by the present methods are also provided. Devices, suitable for testing for an antibody to a fungus, which are produced by the present methods are further provided. In a preferred embodiment, a device for simultaneously testing for a plurality of fungal

5   antibodies is provided, the device comprising a plurality of fungal antigens produced by the present methods attached to a surface of the device suitable for testing for an antibody to a fungus, wherein the plurality of fungal antigens are attached to areas of the surface that are physically distinct from each other. Any suitable device can be used. In one example, the device comprises an immunoblot and wherein the plurality of fungal

10   antigens are attached to separate stripes in the immunoblot. In another example, the device comprises an IFA well and wherein the plurality of fungal antigens are attached to different spots within the IFA well. In still another example, the device comprises an IFA slide and wherein the plurality of fungal antigens are attached to different wells of the IFA slide. Other exemplary devices include a microtiter plate, a glass slide, a

15   nitrocellulose membrane, a latex bead, a cell, a test tube, a plastic bead, a colloidal gold particle, a colored particle, a magnetic bead and a quantum dot.

C.   Methods of testing for an antibody to a fungus

In another aspect, the present invention is directed to a method of testing for an

20   antibody to a fungus in a sample, the method comprising: a) producing a fungal antigen suitable for testing for an antibody to a fungus, comprising providing a fungal antigen as a culture filtrate, the fungal antigen having not been purified by ion exchange chromatography or isoelectric focusing from the culture filtrate, and contacting the fungal antigen with an oxidizing agent to produce an oxidized fungal antigen suitable for testing

25   for an antibody to the fungus; b) contacting a sample suspected of containing an antibody to a fungus with the oxidized fungal antigen produced in step a) under suitable conditions to allow binding of the antibody, if present in the sample, to the oxidized fungal antigen; and c) assessing binding between the antibody and the oxidized fungal antigen to determine the presence and/or amount of the antibody in the sample.

30   The present methods can be used to test for an antibody to any fungus in a sample, including any fungus described in the above Section B. Preferably, the antibody

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to be tested is to a pathogenic fungus such as *Aspergillus, e.g., Aspergillus fumigatus, Blastomyces (Ajellomyces), e.g., Blastomyces dermatitidis, Coccidioides, e.g., Coccidioides immitis, Blastocystis, e.g., Blastocystis hominis, Histoplasma, e.g., Histoplasma capsulatum* and *Candida, e.g., Candida albicans* and *Candida neoformans*.

5   The present methods can be used to test any sample. Preferably, the sample to be tested is a clinical sample including human and veterinary clinical samples. More preferably, the sample to be tested is a human clinical sample.

The oxidized fungal antigens to be used in the present test methods are produced as described in the above Section B. Preferably, the fungal antigen to be oxidized has not 10   been purified from the culture filtrate. Also, preferably, the oxidizing agent to be used is periodate.

The present test methods can be conducted in liquid or solid phase. In specific embodiment, the present test method is conducted in a solid phase the method further comprises a step of attaching the oxidized fungal antigen to a surface of a device suitable 15   for testing for an antibody to a fungus before contacting the antigen with the sample.

The binding between the antibody and the oxidized fungal antigen can be assessed by any suitable assay formats. For example, the binding between the antibody and the oxidized fungal antigen can be assessed by a sandwich or competitive assay format. In another example, the binding between the antibody and the oxidized fungal antigen can 20   be assessed by an enzyme-linked immunosorbent assay (ELISA), immunoblotting, immunoprecipitation, radioimmunoassay (RIA), immunostaining, latex agglutination, indirect hemagglutination assay (IHA), complement fixation, indirect immunofluorescent assay (IFA), nephelometry, flow cytometry assay, chemiluminescence assay, lateral flow immunoassay, u-capture assay, inhibition assay or avidity assay (Manual of Clinical 25   Laboratory Immunology, NR Rose, E Conway de Macario, JD Folds, HC Lane, RM Nakamura, eds. ASM Press, Washington DC, 1997). Preferably, the binding between the antibody and the oxidized fungal antigen is assessed by an ELISA format.

The present assay method can be conducted for prognosis, diagnosis and/or monitoring treatment of pathogenic fungal infection, *e.g.*, histoplasmosis caused by 30   *Histoplasma capsulatum* infection, blastomycosis caused by *Blastomyces dermatitidis* infection, candidasis caused by *Candida albicans* or *Candida neoformans* infection, etc.

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D. Methods for producing an antibody to an fungal antigen

In still another aspect, the present invention is directed to a method for producing an antibody to a fungal antigen, the method comprising: a) producing a fungal antigen comprising providing a fungal antigen as a culture filtrate, the fungal antigen having not been purified by ion exchange chromatography or isoelectric focusing from the culture filtrate, and contacting the fungal antigen with an oxidizing agent to produce an oxidized fungal antigen; b) delivering, to a vertebrate or tissue culture, , the oxidized fungal antigen, in an amount sufficient to induce detectable production of an antibody to the antigen; and c) recovering the antibody from the vertebrate or tissue culture.

The oxidized fungal antigens to be used in the present antibody producing methods are produced as described in the above Section B. Preferably, the fungal antigen to be oxidized has not been purified from the culture filtrate. Also, preferably, the oxidizing agent to be used is periodate.

Any suitable non-human mammal can be used in the present methods. For example, mouse, rabbit and goat can be used.

The antibody to a fungal antigen, *e.g.*, a polyclonal antiserum, which is produced by the present method is also provided.

In yet another aspect, the present invention is directed to a method for producing a monoclonal antibody to an fungal antigen, the method comprising: a) producing a fungal antigen comprising providing a fungal antigen as a culture filtrate, the fungal antigen having not been purified by ion exchange chromatography or isoelectric focusing from the culture filtrate, and contacting the fungal antigen with an oxidizing agent to produce an oxidized fungal antigen; b) delivering, to a vertebrate or tissue culture, the oxidized fungal antigen, in an amount sufficient to induce detectable production of an antibody to the antigen; c) removing at least a portion of antibody-producing cells from the vertebrate or tissue culture; d) immortalizing the removed antibody-producing cells; e) propagating the immortalized antibody-producing cells; and f) harvesting monoclonal antibody produced by the immortalized antibody-producing cells. Methods of testing for a fungal antigen in a sample using the produced monoclonal antibodies are also provided.

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The oxidized fungal antigens to be used in the present antibody producing methods are produced as described in the above Section B. Preferably, the fungal antigen to be oxidized has not been purified from the culture filtrate. Also, preferably, the oxidizing agent to be used is periodate.

5   Any suitable non-human mammal can be used in the present methods. For example, mouse, rabbit and goat can be used.

A monoclonal antibody to a fungal antigen, which is produced by the present method is also provided.

A hybridoma capable of producing a monoclonal antibody to a fungal antigen,
10   which is produced by steps a)-d) of the present method is further provided.

The oxidized fungal antigens can be delivered to a vertebrate or non-human mammal by any methods known in the art (*See e.g.,* Coligan et al. (Ed.), Current Protocols in Immunology, 2.II Production of Antibodies, John Wiley & Sons, Inc. (2000)).

15   The oxidized fungal antigens can be delivered to the interstitial space of tissues of the animal body, including those of muscle, skin, brain, lung, liver, spleen, bone marrow, thymus, heart lymph, blood, bone, cartilage, pancreas, kidney, gall bladder, stomach, intestine, testis, ovary, uterus, rectum, nervous system, eye, gland, and connective tissue. Interstitial space of the tissues comprises the intercellular, fluid, mucopolysaccharide
20   matrix among the reticular fibers or organ tissues, elastic fibers in the walls of vessels or chambers, collagen fibers of fibrous tissues, or that same matrix within connective tissue ensheathing muscle cells or in the lacunae of bone. It is similarly the space occupied by the plasma of the circulation of the lymph fluid of the lymphatic channels.

The oxidized fungal antigens can be conveniently delivered by injection into the
25   tissues comprising these cells. They are preferably delivered to persistent, non-dividing cells which are differentiated, although delivery can be achieved in non-differentiated or less completely differentiated cells, such as, for example, stem cells of blood or skin fibroblasts.

In a specific embodiment, the oxidized fungal antigen is delivered directly to a
30   tissue of the animals. Preferably, the oxidized fungal antigen is delivered directly to muscle, skin or mucous membrane. In one example, the oxidized fungal antigen can be

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delivered directly to a tissue of the animal by injection, by gene gun technology or by lipid mediated delivery technology. The injection can be conducted via a needle or other injection devices. The gene gun technology is disclosed in U.S. Patent No. 5,302,509 and the lipid mediated delivery technology is disclosed in U.S. Patent No. 5,703,055, the

5   contents of which are incorporated herein by reference.

In still another specific embodiment, the oxidized fungal antigen is delivered to a cell of the animal and the cell containing the oxidized fungal antigen is delivered to a suitable tissue of the animal. Preferably, the oxidized fungal antigen is delivered to a blood cell of an animal. More preferably, the oxidized fungal antigen is delivered to a

10   spleen B cell of an animal.

The anti-fungal antibodies, whether polyclonal or monoclonal, that are produced by the above methods can be used in testing for a fungal antigen in a sample. In one specific embodiment, the present invention is directed to a method of testing for a fungal antigen in a sample, the method comprising: a) providing an anti-fungal polyclonal

15   antiserum produced by the above antibody producing method; b) contacting a sample suspected of containing a fungal antigen with the polyclonal antiserum under suitable conditions to allow binding of the fungal antigen, if present in the sample, to the polyclonal antiserum; and c) assessing binding between the fungal antigen and the polyclonal antiserum to determine the presence and/or amount of the fungal antigen in

20   the sample.

In another specific embodiment, the present invention is directed to a method of testing for a fungal antigen in a sample, the method comprising: a) providing an anti-fungal antigen monoclonal antibody produced by the above antibody producing method; b) contacting a sample suspected of containing a fungal antigen with the monoclonal

25   antibody under suitable conditions to allow binding of the fungal antigen, if present in the sample, to the monoclonal antibody; and c) assessing binding between the fungal antigen and the monoclonal antibody to determine the presence and/or amount of the fungal antigen in the sample.

30   **E.   Exemplary embodiments**

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In exemplary embodiments of the present invention, oxidized preparations of crude fungal antigens are used in an ELISA format, resulting in the sensitive and specific detection of fungal antibodies. The fungal antigens are oxidized during a short (2-hour) incubation with periodate, then further diluted with coating buffer and allowed to adsorb

5 to polystyrene microtiter wells. ELISA performance using these coated microtiter wells is complete within 4 hours, thus providing sensitive and specific results more quickly and easily than ID and western blot assays.

Preparation of antigen-coated microtiter wells

10 A fungal antigen preparation, designed for use in ID assays, is purchased from a commercial source. The protein concentration of the antigen preparation is determined by protein-dye binding (Bradford) assay. The fungal antigen is then diluted to a final protein concentration of about 10-50 micrograms per milliliter (ug/mL) in 0.05M sodium acetate buffer (adjusted to pH 5.5 using 10% acetic acid) containing about 0.02M-0.10M

15 sodium meta-periodate. After a short incubation period (1-3 hours) at room temperature, during which hydroxyl groups of the fungal antigen carbohydrate moieties are cleaved and converted to aldehydes (i.e., become oxidized), the oxidized fungal antigen preparation is diluted approximately 20-fold with phosphate buffered saline (PBS), pH 7.4. This diluted fungal antigen is then added to polystyrene microtiter wells at a volume

20 of 0.1 mL per well. The microtiter wells are covered with plastic adhesive tape and incubated in the refrigerator (4-8°C) for 14-18 hours. During this incubation, fungal antigen protein moieties attach to the polystyrene through hydrophobic interactions. After incubation, the solution is discarded from the microtiter wells, now bearing attached oxidized fungal antigen. All microtiter wells then receive 0.2 mL of PBS

25 containing a blocking agent, such as bovine serum albumin (BSA), and a stabilizing agent, such as trehalose. The blocking agent attaches to any polystyrene binding sites that are left exposed after the incubation with oxidized fungal antigen. This step reduces the nonspecific attachment of antibody molecules not directed toward the specific antigens, and also reduces nonspecific adsorption of the conjugate during the color

30 generation step. After 2 hours at room temperature, the blocking solution is discarded from the microtiter wells. The microtiter plates are air-dried at room temperature, then

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placed inside a plastic pouch, and the pouch heat-sealed. Sealed pouches containing microtiter plates coated with fungal antigens are stored in the refrigerator until use in the ELISA.

ELISA performance

Antigen-coated microtiter well plates are removed from the refrigerator and allowed to warm to room temperature. The wells are then filled with PBS containing a mild detergent such as Tween 20 (PBST), and after 5 minutes this PBST is discarded. This wash step ensures that all antigen and blocking solution proteins have been removed; in addition, the small amount of detergent helps to reduce bubble formation in subsequent steps. Serum samples are diluted in PBST that contains the same protein that is used as a blocking agent (such as BSA). About 0.1 mL of each diluted serum is added to an assigned microtiter well. The microtiter plate is then covered with adhesive tape and incubated about 1.5 hours at room temperature. During this incubation period, any serum antibodies directed to the antigen that is attached to the microtiter well will bind to the antigen. At the end of this incubation, the liquid material is discarded from all the wells. About 0.25 mL of PBST is then added to each well, and this fluid is then discarded; this wash step is repeated three times. This washing procedure ensures that non-attached serum components are removed from the microtiter well. About 0.1 mL of a conjugate diluted in PBST is then added to each well. This conjugate is a commercially-available, species-specific, anti-immunoglobulin with an enzyme chemically linked (conjugated) to it. The enzyme used is selected based on its ability to react with a substrate to produce a colored compound. A typical conjugate would be goat anti-human IgG tagged with horseradish peroxidase. This conjugate will bind to any IgG antibodies in the serum that attached to the antigen attached to the microtiter well. Following another series of washes to remove any unbound conjugate, enzyme substrate is added to each microtiter well. A typical substrate would be tetra-methylbenzidine (TMB) and hydrogen peroxide; in the presence of horseradish peroxidase, this substrate produces a bluish purple color. Thus, color only develops when a "sandwich" consisting of plastic-bound antigen, serum-derived antibody, and enzyme-tagged conjugate has been built up; if the serum contains no antigen-specific antibody molecules, then the enzyme-

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tagged conjugate will not bind, and no color will develop because the enzyme is absent from the system. After a defined amount of time, a solution of dilute hydrochloric acid, or some other acid, is added to lower the pH of the solution in the wells. At a lower pH, the bluish purple color changes to a yellow color. The absorbance (also called optical density) of the yellow color is then quantitated using a spectrophotometric instrument. The microtiter well plate is placed inside the instrument, and the optical density for each well (i.e., each sample) is measured at a light wavelength of 450 nanometers. The optical density value is directly proportional to the intensity of the yellow color, which in turn is directly proportional to the amount of antigen-specific IgG antibody bound to the well.

EXAMPLE 1: Detection of IgG antibodies recognizing *Histoplasma capsulatum*

Histoplasma capsulatum antigen (culture filtrate containing both H and M antigens) intended for use in ID assays was purchased from a commercial source, and adjusted to 20 ug/mL in sodium acetate buffer containing 0.05M periodate (hereafter referred to as oxidized antigen). For comparative purposes, a duplicate antigen suspension in sodium acetate buffer without periodate was prepared (hereafter referred to as nonoxidized antigen). After 2 hours at room temperature, the antigen preparations were diluted 20-fold in PBS to a final concentration of 1 ug/mL, then added to microtiter wells (0.1 mL per well). Thus, some microtiter wells received oxidized Histoplasma antigen, whereas other wells received nonoxidized Histoplasma antigen. After an overnight incubation in the refrigerator, the Histoplasma antigen preparations (oxidized or nonoxidized) were discarded from the wells, and PBS containing 0.1% (w/v) BSA and 5% (w/v) trehalose was added to the wells as blocking agent. After 2 hours at room temperature, the blocking agent was discarded, and the plates were then air-dried at room temperature for 2 hours. After pouching, the plates were stored in the refrigerator.

On the day of assay, Histoplasma-coated microtiter wells (some coated with oxidized antigen, others coated with nonoxidized antigen) were allowed to come to room temperature. During this step, 80 serum samples previously tested for Histoplasma antibodies by ID (16 positive, 64 negative) were diluted 1:400 in sample dilution buffer (PBST containing BSA). Once the wells had reached room temperature (approximately 1 hour), they were washed once with PBST, and the wash solution discarded. Diluted

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serum samples were then added to duplicate microtiter wells (one containing oxidized Histoplasma antigen, the other containing nonoxidized Histoplasma antigen). The microtiter wells were covered with adhesive tape and incubated at room temperature for 1.5 hours. Following 4 washes with PBST, each well then received 0.1 mL of

5 horseradish peroxidase-conjugated goat anti-human IgG (diluted 1:10000 in PBST), and incubation was continued for 1 hour at room temperature. Following another series of 4 washes, each well then received 0.1 mL of tetra-methylbenzidine; after 10 minutes at room temperature, the reaction was stopped by the addition of 0.1N hydrochloric acid. The optical density was measured at 450 nM using a spectrophotometric ELISA reader.

10 Figure 1 presents the optical density (OD) values obtained following incubation of ID-positive and ID-negative serum samples in microtiter wells coated with nonoxidized and oxidized Histoplasma antigen. When using nonoxidized Histoplasma antigen as substrate, the distribution of OD values for the ID-negative serum group was similar to that observed for the ID-positive serum group. In contrast, when using the

15 oxidized Histoplasma antigen as substrate, the distribution of values for the ID-negative serum group was markedly different from the distribution of values for the ID-positive serum group. When using the oxidized antigen, 16 of 16 (100%) ID-positive serum samples exhibited OD values >1.45, whereas only 4 of 64 (6%) ID-negative serum samples exhibited OD values >1.45. Expressed another way, the Histoplasma IgG assay

20 using oxidized Histoplasma antigen as substrate was 100% sensitive and 94% specific (see Table 2) using an OD cutoff value of 1.45.

Table 2.   Specificities of fungal IgG ELISAs using oxidized versus nonoxidized antigens under conditions giving the same sensitivities

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ELISA	Antigen type	Cutoff OD	Sensitivity (%)	Specificity (%)
Histoplasma	Oxidized	1.45	100	94
IgG	Nonoxidized	0.50	100	25
Coccidioides	Oxidized	1.00	90	97
IgG	Nonoxidized	0.22	90	28
Aspergillus	Oxidized	.0.85	89	95
IgG	Nonoxidized	0.23	89	65
Blastomyces	Oxidized	0.40	100	100
IgG	Nonoxidized	0.50	100	87

For comparative purposes, we determined the specificity of the Histoplasma IgG assay using nonoxidized antigen under conditions where the sensitivity using nonoxidized antigen matched that seen using oxidized antigen (i.e., 100%). Thus, the cutoff OD giving 100% sensitivity when using nonoxidized antigen was 0.50 (Figure 1). Based on this cutoff OD value, the specificity of the Histoplasma IgG assay using nonoxidized antigen was only 25% (see Table 2). Thus, under conditions providing 100% sensitivity, oxidation of Histoplasma antigen profoundly improved the specificity of an ELISA for detecting Histoplasma IgG.

EXAMPLE 2: Detection of IgG antibodies recognizing *Coccidioides immitis*

Coccidioides immitis antigen (culture filtrate containing F antigen) intended for use in ID assays was purchased from a commercial source. Microtiter well preparation and assay performance was the same as described in Example 1 for Histoplasma, with the exception that conjugate was used at a final dilution of 1:5000 rather than 1:10000. The serum panel evaluated for Coccidioides IgG consisted of 80 samples previously tested for Coccidioides antibodies by ID (41 positive, 39 negative).

Figure 2 presents the optical density (OD) values obtained following incubation of ID-positive and ID-negative serum samples in microtiter wells coated with nonoxidized and oxidized Coccidioides antigen. When using nonoxidized Coccidioides antigen as substrate, the distribution of OD values for the ID-negative serum group was similar to that observed for the ID-positive serum group. In contrast, when using the oxidized Coccidioides antigen as substrate, the distribution of values for the ID-negative serum group was markedly different from the distribution of values for the ID-positive serum group. When using the oxidized antigen, 37 of 41 (90%) ID-positive serum samples exhibited OD values >1.00, whereas only 1 of 39 (3%) ID-negative serum samples exhibited OD values >1.00. Expressed another way, the Coccidioides IgG assay using oxidized Coccidioides antigen as substrate was 90% sensitive and 97% specific (see Table 2) using an OD cutoff value of 1.00.

For comparative purposes, we determined the specificity of the Coccidioies IgG assay using nonoxidized antigen under conditions where the sensitivity using nonoxidized antigen matched that seen using oxidized antigen (i.e., 90%). Thus, the

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cutoff OD giving 90% sensitivity when using nonoxidized antigen was 0.22 (Figure 2). Based on this cutoff OD value, the specificity of the Coccidioides IgG assay using nonoxidized antigen was only 28% (see Table 2). Thus, under conditions providing 90% sensitivity, oxidation of Coccidioides antigen profoundly improved the specificity of an

5   ELISA for detecting Coccidioides IgG.

EXAMPLE 3: Detection of IgG antibodies recognizing *Aspergillus fumigatus*

Aspergillus fumigatus antigen (culture filtrate) intended for use in ID assays was purchased from a commercial source. Microtiter well preparation and assay performance

10   was the same as described in Example 1 for Histoplasma, with the exception that conjugate was used at a final dilution of 1:5000 rather than 1:10000. The serum panel evaluated for Aspergillus IgG consisted of 29 samples previously tested for Aspergillus antibodies by ID (9 positive, 20 negative).

Figure 3 presents the optical density (OD) values obtained following incubation

15   of ID-positive and ID-negative serum samples in microtiter wells coated with nonoxidized and oxidized Aspergillus antigen. For both nonoxidized and oxidized antigen, the overall distribution of OD values for the ID-negative group was visually distinct from the distribution of OD values for the ID-positive group. However, the range of difference between the ID-negative distribution and the ID-positive distribution was

20   more marked when using oxidized Aspergillus antigen. When using the oxidized antigen, 8 of 9 (89%) ID-positive serum samples exhibited OD values >0.85, whereas only 1 of 20 (5%) ID-negative serum samples exhibited OD values >0.85. Expressed another way, the Aspergillus IgG assay using oxidized Aspergillus antigen as substrate was 89% sensitive and 95% specific (see Table 2) using an OD cutoff value of 0.85.

25   For comparative purposes, we determined the specificity of the Aspergillus IgG assay using nonoxidized antigen under conditions where the sensitivity using nonoxidized antigen matched that seen using oxidized antigen (i.e., 89%). Thus, the cutoff OD giving 89% sensitivity when using nonoxidized antigen was 0.23 (Figure 3). Based on this cutoff OD value, the specificity of the Aspergillus IgG assay using

30   nonoxidized antigen was 65% (see Table 2). Thus, under conditions providing 89%

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sensitivity, oxidation of Aspergillus antigen markedly improved the specificity of an ELISA for detecting Aspergillus IgG.

EXAMPLE 4: Detection of IgG antibodies recognizing *Blastomyces dermatitidis*

5   *Blastomyces dermatitidis* antigen (culture filtrate containing A antigen) intended for use in ID assays was purchased from a commercial source. Microtiter well preparation and assay performance was exactly the same as described in Example 1 for Histoplasma. The serum panel evaluated for Blastomyces IgG consisted of 16 samples previously tested for Blastomyces antibodies by ID (1 positive, 15 negative).

10   Figure 4 presents the optical density (OD) values obtained following incubation of ID-positive and ID-negative serum samples in microtiter wells coated with nonoxidized and oxidized Blastomyces antigen. When using the oxidized antigen, the single ID-positive serum sample exhibited an OD value >0.4, whereas only 0 of 15 (0%) ID-negative serum samples exhibited OD values >0.40. Expressed another way, the

15   Blastomyces IgG assay using oxidized Basltomyces antigen as substrate was 100% sensitive and 100% specific (see Table 2) using an OD cutoff value of 0.40.

        For comparative purposes, we determined the specificity of the Blastomyces IgG assay using nonoxidized antigen under conditions where the sensitivity using nonoxidized antigen matched that seen using oxidized antigen (i.e., 100%). Thus, the

20   cutoff OD giving 100% sensitivity when using nonoxidized antigen was 0.50 (Figure 4). Based on this cutoff OD value, the specificity of the Blastomyces IgG assay using nonoxidized antigen was 87% (see Table 2). Thus, under conditions providing 100% sensitivity, oxidation of Blastomyces antigen improved the specificity of an ELISA for detecting Blastomyces IgG.

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        The above examples are included for illustrative purposes only and are not intended to limit the scope of the invention. Many variations to those described above are possible. Since modifications and variations to the examples described above will be apparent to those of skill in this art, it is intended that this invention be limited only by

30   the scope of the appended claims.